V. A. Motsavage and H. B. Kostenbauder, *J. Colloid Sci.*, **18**, 603 (1963); C. J. O'Connor, E. J. Fendler, and J. H. Fendler, *J. Chem. Soc., Perkin Trans. 2*, 1900 (1973); or (b) the catalysis is due to a "solvent effect", e.g., M. D. Bentley, S. E. Bowie, and R. D. Limogenes, *J. Phys. Chem.*, **75**, 1763 (1971); the neutral hydrolysis of tosylmethyl perchlorate in water (without CTABr) is rather independent of the dielectric constant of the medium.[10a]

(16) M. J. Minch, M. Giaccio, and R. Wolff, *J. Am. Chem. Soc.*, **97**, 3766 (1975).

(17) It is questionable how much the strong decrease of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ in 0.1 N HCl + 2.0×10^{-3} M CTABr could be the result of an electrolyte effect on the formation of micelles or on the micellar catalysis. Electrolyte effects generally decrease the magnitude of the catalysis.[1-5]

(18) C. A. Bunton and L. Robinson, *J. Am. Chem. Soc.*, **90**, 5972 (1968).

(19) (a) C. A. Bunton and L. Robinson, *J. Org. Chem.*, **34**, 773 (1969); (b) C. A. Bunton, L. Robinson, and L. Sepulveda, *J. Am. Chem. Soc.*, **91**, 4813 (1969).

(20) P. Mukerjee, P. Kapauan, and H. G. Meyer, *J. Phys. Chem.*, **70**, 783 (1966); G. C. Kresheck, H. Schneider, and H. A. Scheraga, *J. Phys. Chem.*, **69**, 3132 (1965).

(21) Furthermore, micelles in D_2O could be more effective than those in water, because of stronger hydrophobic interactions. Previous investigations by other workers, however, do not support this view: C. A. Bunton and S. Diaz, *J. Org. Chem.*, **41**, 33 (1976).

(22) Private communication from L. Menninga.

(23) Addition of small amounts (10^{-5}–10^{-4} M) of compounds structurally related to the substrate, e.g., tosylmethyl nitrate or tosylmethane, which did not hydrolyze appreciably under the reaction conditions employed, had no effect on the rate.

(24) Acetonitrile or methanol also can be used with the same results.

(25) D. de Filippo and F. Momicchiolo, *Tetrahedron*, **25**, 5733 (1969).

(26) The $HCOO^-$-catalyzed hydrolysis of **1** in CTABr micelles was performed at an initial pH of ca. 4 and a constant ion strength of 4.1×10^{-3} M (by addition of NaCl). Further experiments without NaCl revealed, however, that k_{HCOO^-} and k_{Ts^-} were not influenced by the differences in ionic strength.

(27) We assume that the association constants of **1** and *p*-toluenesulfinic acid are large, due to the hydrophobicity of the tolyl group.[6] Unfortunately, the complexity of the CTABr-catalyzed reaction strongly hampers the determination of a reliable binding constant for **1** from the kinetic data.

(28) C. A. Bunton and M. J. Minch, *J. Phys. Chem.*, **78**, 1490 (1974).

(29) (a) A plot of ($k_{obsd} - A$) vs. concentration of hydroxide ions during the reaction shows almost a linear correlation. From the slope of this plot we tentatively conclude that pseudo-first-order kinetics may be expected if k_{OH^-} is about 10^8 M^{-1} s^{-1}. In water in the absence of CTABr, k_{OH^-} has recently been determined as 6.4×10^4 M^{-1} s^{-1}.[22] If the acceleration of the hydroxide ion catalyzed reaction is comparable to that for formate and *p*-toluenesulfinate, a value of 10^8 is reasonable indeed. There are strong indications, however, that the use of k_{Ts^-} and k_{HCOO^-} as determined in separate experiments is questionable. We found that at substrate concentrations >7–8×10^{-5} M the sum of the calculated contributions of sulfinate and formate exceeds k_{obsd}. Therefore the actual contributions of these species are smaller than calculated, which points to a competition of these anions for "active sites" (see also ref 19a). (b) A referee has pointed out that the situation is further complicated by the unknown distribution of acid and anion between water and the micelle. We feel, however, that this complexity (reflected in k_{Ts^-} and k_{HCOO^-}) does not affect our explanation in terms of compensatory kinetic effects.

(30) The effect of stirring on the rate depends on the stirrer and the rate of stirring. Absolute values of reaction rate with stirring have therefore little meaning. The fluctuations in the effect are in the order of ca. ±10%, probably due to uncontrolled variations in stirring speed. The effect also was observed if the reaction was performed in a glass vessel and followed by conductivity measurements. A similar effect of stirring was found also for *p*-nitrophenylsulfonylmethyl perchlorate.

(31) It is well known that aggregation of polymers may be assisted by orientation of the molecules in a hydrodynamic field. See, for example: S. Frenkel, *J. Polym. Sci., Polym. Symp.*, **No. 44**, 49 (1974). Micellization might be induced likewise by orientation of the surfactant molecules in the direction of the stirring stream.

(32) Support for this type of effect is found in the decrease of surface tension upon stirring as reported by: C. W. Brown, D. Cooper, and J. C. S. Moore, *J. Colloid. Sci.*, **32**, 584 (1970).

(33) I. J. Lin and P. Somasundaran, *J. Colloid. Sci.*, **37**, 731 (1971).

(34) P. Somasundaran and D. W. Fuerstenau, *J. Phys. Chem.*, **70**, 90 (1966); P. Somasundaran, T. W. Healy, and D. W. Fuerstenau, *J. Phys. Chem.*, **68**, 3562 (1964).

(35) F. Menger, *J. Am. Chem. Soc.*, **92**, 5965 (1970); F. Menger in "Water, A Comprehensive Treatise", Vol. 3, F. Franks, Ed., Plenum Press, New York, N.Y., 1973, p 229.

(36) Hemimicelle formation itself might also be pH dependent, see ref 34.

(37) There is evidence for a relatively high hydroxide ion concentration at the interface. See, for example, ref 34 and H. A. Zutrauen, *J. Chim. Phys.*, **53**, 54 (1956).

Kinetics and Mechanisms of the 1,5-Dihydroflavin Reduction of Carbonyl Compounds and the Flavin Oxidation of Alcohols. 4. Interconversion of Formaldehyde and Methanol

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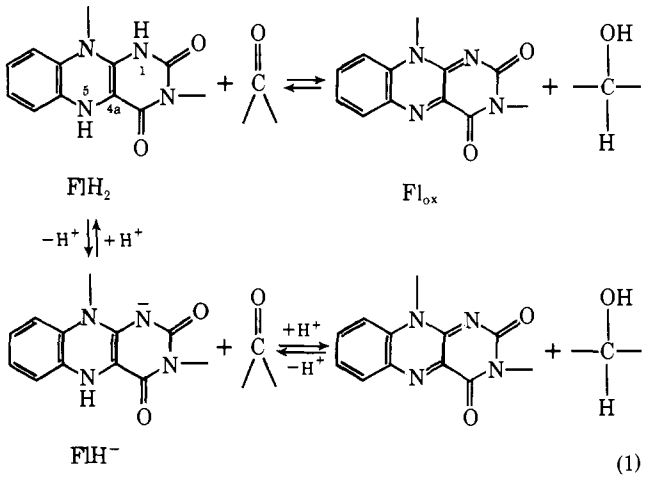
Abstract: The mechanism of the kinetically biphasic reduction of formaldehyde by dihydroflavin (ref 9) has been elucidated. In the first and rapid phase, dihydroflavin and formaldehyde yield, via two competing reactions: (a) oxidized flavin plus methanol; and (b) the $N(5)$-hydroxymethyl derivative plus the corresponding imine. In the slower second phase of reaction the oxidized flavin produced in the first phase acts as a catalyst in the conversion of the $N(5)$-hydroxymethyl derivative to reduced flavin plus formaldehyde, and these reactants reenter the reactions of the first phase. Concentration and pH dependencies have been investigated and the appropriate rate constants determined. 1,5-Dihydro-3-methyllumiflavin is proposed to form an $N(1)$-hydroxymethyl adduct as has been shown (ref 25) for uracils. Methanol has been shown to reduce oxidized flavin at alkaline pH providing as products 1,5-dihydroflavin and formaldehyde in a 1:1 ratio. Previous studies are reviewed (Introduction) and a general radical mechanism presented (Discussion).

Introduction

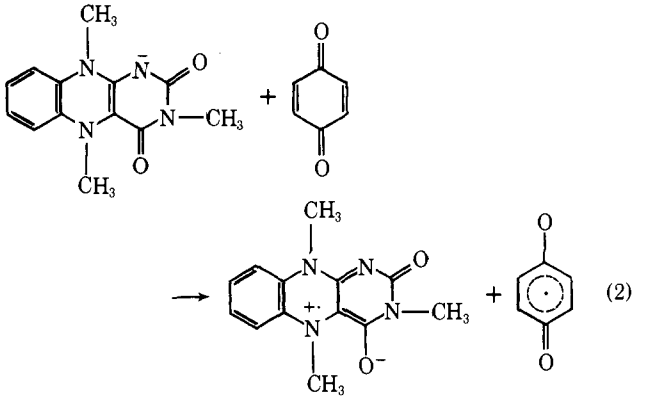
The elucidation of the mechanisms of flavin mediated oxidation–reduction reactions in simple chemical systems is of prime importance to the understanding and appreciation of the mechanisms of catalysis by flavoenzymes. In those examples of enzymatic reactions where an organic cofactor is required, physical organic studies of the mechanism of reaction of the cofactor with substrate (sans apoenzyme) have proved invaluable to the understanding of the enzyme catalytic process. Even appreciation of the differences in the mechanisms of model reactions and enzyme reactions are of importance since they explicitly describe the role of the apoenzyme. The present study is the fourth[2-4] in a series dealing with the kinetics and mechanisms for the overall reactions of eq 1.

In the two-electron oxidation (or reduction) of one organic



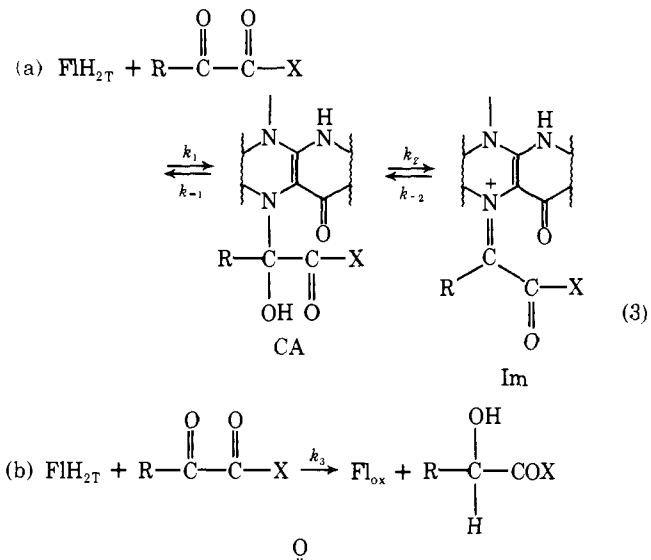
(1)

compound by another, the question of mechanism revolves around the means by which the electrons are transferred. It has been established[2] that reaction of *N*(5)-methyl-1,5-dihydrolumiflavin with quinones, triketohydrindane hydrate etc. provides the flavin aminium cation radical (eq 2). Thus, $1e^-$



(2)

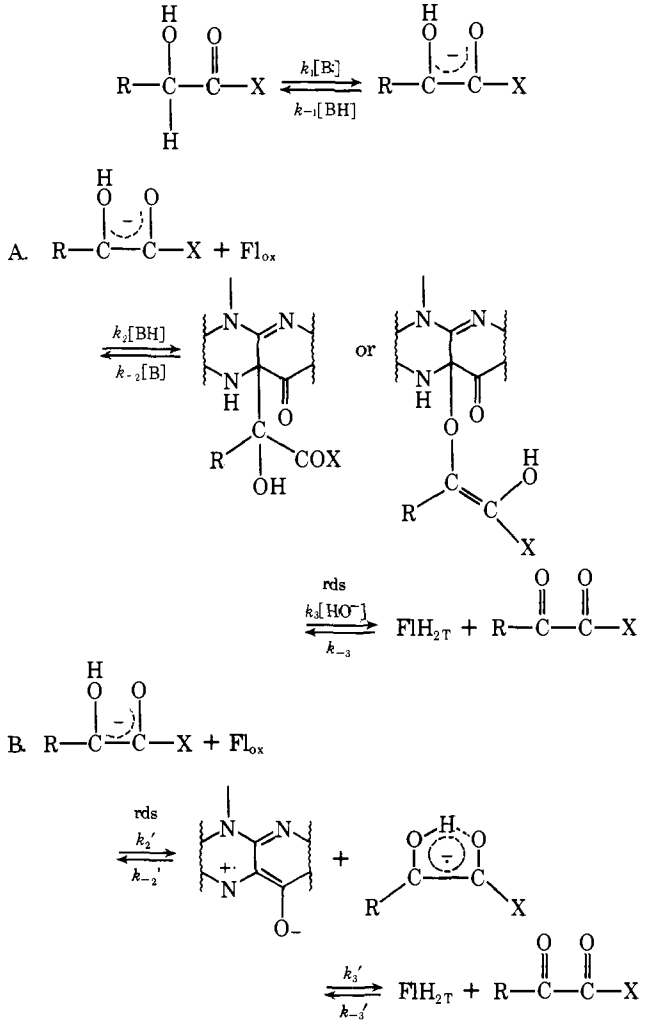
transfer from dihydroflavin to carbonyl oxidant is favored in these examples wherein the radical products possess considerable thermodynamic stability. Radicals generated by $1e^-$ transfer to α-diketones also exhibit considerable thermodynamic stability due to their semidione structure. Thus, it might be anticipated that the 1,5-dihydroflavin reduction of compounds of general structure R-C(=O)-C(=O)X or the flavin oxidation of compounds of general structure R-CH(OH)-C(=O)X might proceed through $1e^-$ transfer reactions. The reduction of pyruvic acid, pyruvate, pyruvamide, and ethyl pyruvate at given constant pH and buffer concentration has been quantitatively established to follow the kinetic steps of eq 3.[3] In eq 3 conversion of the pyruvate species (R = CH_3-, X = $-OC_2H_5$, $-NH_2$, -OH, and $-O^-$) to the corresponding lactate occurs in a reaction first order in $[FlH_2]_T$ and $[CH_3CO\text{-}COX]$ which is catalyzed by pyruvic acid and H_3O^+. Formation of carbinolamine (CA) and imine (Im) occur but only through nonproductive equilibria. The kinetics for the appearance of Fl_{ox} on reduction of benzil by FlH_{2T} (at constant buffer concentration and pH) resemble that for the reduction of pyruvate by FlH_{2T}.[4] Presumably, the reaction sequence of eq 3 (R = X = $-C_6H_5$) applies to this reaction as well. The reduction of Fl_{ox} by α-ketols (benzoin, lactamide, and ethyl lactate) has also been established.[3,4] In the case of benzoin the reaction has been shown[2] to proceed through general base catalyzed ionization of the α-CH proton followed by a non-acid-base catalyzed reaction of the resultant endiolate ion with Fl_{ox} to yield FlH_{2T} and benzil. If one assumes for this overall $2e^-$ transfer reaction that an intermediate (radical pair or covalent adduct) is a requisite, then the determined kinetics find mechanistic interpretation in either the 4a-addition



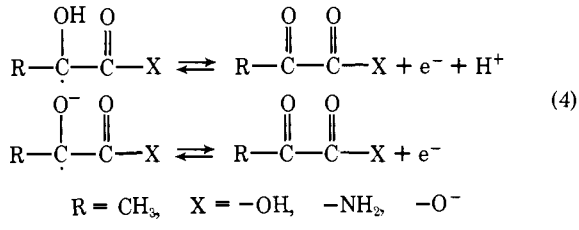
(3)

mechanism of Scheme IA or the radical mechanism of Scheme IB [a mechanism involving addition of carbanion to the N(5)

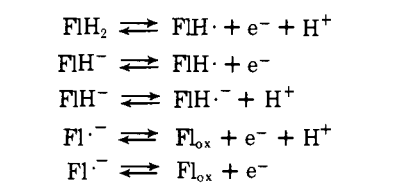
Scheme I



position (i.e., carbinolamine) is also allowed in this case]. These mechanisms cannot be kinetically differentiated. However, based on $E^{\circ\prime}$ values[5,6] for the half-reactions of eq 4 and the E° values[7] for eq 5, there may be computed the standard (1 M, designated pH) free energies of formation of the radical species

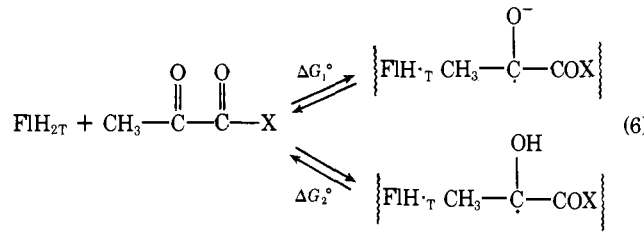


(4)



(5)

of eq 6 in the pH range of interest ($FlH\cdot_T$ = total flavin radical species). For pyruvic acid, pyruvate, pyruvamide, and ethyl



(6)

pyruvate $\Delta G_1°$ and $\Delta G_2°$ were found to be less positive by 9–11 kcal M^{-1} than the experimentally determined values of $\Delta G^{\ddagger}$ for dihydroflavin reduction of these substrates. Thus, radical pair intermediates are eminently reasonable (k_3 of Scheme I) for dihydroflavin reduction of pyruvate derivatives. Since the values of $\Delta G_1°$ and $\Delta G_2°$ for the formation of anion and neutral radicals from FlH_{2T} and benzoin should be even less positive, we may feel assured that radical intermediates are also most reasonable in the flavin mediated interconversion of benzoin and benzil (Scheme IB). The Fl_{ox} mediated dehydrogenation of *trans*-dimethyl dihydrophthalate has been shown (like benzoin) to occur by general base catalyzed formation of carbanion which then reacts with Fl_{ox} in a non-acid-base catalyzed reaction.[8] Mechanisms completely analogous to those of Schemes IA, B may be written for this reaction as well.

The mechanisms of Schemes IA,B require that a carbon acid dissociate to yield a resonantly stabilized carbanion which is then oxidized to ketone. These mechanisms cannot apply to the reduction of Fl_{ox} by very weak carbon acids. We have previously shown that 1,5-dihydroflavin readily reduces formaldehyde to methanol.[9,10] In the present study it is established that $CH_3OH + Fl_{ox} \rightarrow CH_2O + FlH_2$. The $\Delta G°$ value for $CH_3OH \rightarrow H^+ + {}^-CH_2OH$ exceeds $\Delta G^{\ddagger}$ so that carbanion formation is disallowed. The kinetics for the reduction of formaldehyde by 1,5-dihydroflavin are described and a general mechanism proposed.

Experimental Section

Materials. 3-Carboxymethyl-7,8,10-trimethylisoalloxazine (I, lumiflavin-3-acetate) was synthesized and purified as previously described.[3] **3,7,8,10-Tetramethylisoalloxazine (II**, 3-methyllumiflavin) was prepared according to the method of Hemmerich.[11] Carbonyl compounds employed as substrates were products of the following companies and were used without further purification: formaldehyde (Mallinckrodt), pyridoxal hydrochloride (Mann Research Lab.), pyridine-4-carboxaldehyde, oxalacetic acid, glyoxylic acid hydrate, glycoaldehyde (Aldrich), glycolamide (Sigma), and glyoxal (Matheson, Coleman, and Bell). Phenylglyoxal (K & K Laboratories) was recrystallized from water after treatment with charcoal, mp 74–76 °C (uncorrected). Sodium cyanoborohydride (Aldrich) was purified according to the procedures outlined by Borch et al.[12] and stored under moisture-free nitrogen. Methanol (Mallinkrodt) was purified by distillation immediately before use. Formaldehyde solutions were also prepared by pyrolysis of paraformaldehyde (Aldrich) according to the method of Gilman and Catlin.[13] Formaldehyde gas was trapped by an aqueous solution (doubly glass distilled water) containing 10 vol % methanol as a stabilizer. Concentrations of stock formaldehyde solutions were determined by titration with sodium sulfite.[14] All other salts and buffer materials were analytical reagent grade and were used without further purification.

Methods. Methods and anaerobic kinetic techniques have been previously described.[3] Temperature control was maintained at 30.0 ± 0.1 °C for all kinetic measurements. Measurements of pH were made before and after each kinetic run (pH drifts of >0.02 unit were discarded and repeated). Polarographic measurements were made on a Model 174 Princeton Applied Research polarographic analyzer equipped with a saturated calomel reference electrode thermostated at 25.0 °C. All formaldehyde solutions were deoxygenated by bubbling vanadous scrubbed argon or nitrogen before their concentrations were determined by titration. After deoxygenation the formaldehyde solutions were stored in a nitrogen atmosphere gloved-box to maintain anaerobicity. Anaerobic Thunberg cuvettes were charged with the desired aliquots of stock formaldehyde solutions in the nitrogen box. The following buffers were employed to maintain constant pH: α-chloroacetic acid and KOH for pH <3.5, potassium acetate and acetic acid for pH 3.5–5.5, K_2HPO_4 and KH_2PO_4 for pH 5.5–7.5, boric acid and KOH for pH 7.5–9.0, potassium carbonate and KOH for pH >9.0.

Product Analysis for the Reduction of Lumiflavin-3-acetate (I) by Methanol. Typically, three Thunberg cuvettes were prepared containing 0.5 ml of a stock solution of I (6.4×10^{-4} M) in methanol plus 0.0, 0.5, or 0.75 ml of 1 M KCl in the top port and 2.5 ml of a 0.1 M carbonate buffer (μ = 1.0 with KCl, pH 9.65) in the bottom. After careful deoxygenation by bubbling vanadous ion scrubbed argon for 45–60 min through each port, the Thunbergs were transferred to an anaerobic (nitrogen atmosphere) gloved-box. The top and bottom were mixed, and 0.25, 0.5, or 1.0 ml of methanol was rapidly placed into the top port. The cuvette was removed from the anaerobic box and scanned to determine the initial I absorbance. Following temperature equilibration (30.0 °C) in the Cary 16, the contents of each port were mixed to give a solution of final concentration: [I] = 8.0×10^{-5} M; [MeOH] = 1.53, 3.06, or 6.12 M. The disappearance of I was followed at λ 443 nm for 8½ days. Oxygen was introduced into the cuvette and the absorbance increase at 443 nm produced by oxidation of the 1,5-dihydrolumiflavin-3-acetic acid (IH_2) was measured. A blank reaction, containing no added methanol (pH 10.9, 0.1 M carbonate, μ = 1.0 with KCl), showed only hydrolysis of I with no absorbance increase on addition of oxygen. Similar experiments were repeated using higher concentrations of I (1.03×10^{-3} or 8.30×10^{-4} M) and methanol (9.2 M). Upon completion of the reaction the solutions were analyzed for formaldehyde content by differential pulse polarographic analysis employing a dropping mercury electrode. Best results were achieved when the reaction solutions were adjusted to pH 12.3 with base before determination of the $E_{1/2}$. Standard solutions containing all components (under conditions identical with the reaction mixture) were prepared with no formaldehyde or known amounts of formaldehyde ($[CH_2O] = 10^{-6}$ to 10^{-3} M). The determined $E_{1/2}$ of −1.67 V for formaldehyde agreed well with published values.[16] The concentration of formaldehyde in the reaction mixtures was determined from the current (μamp) vs. concentration relationship.

Results

The kinetics for oxidation of 1,5-dihydrolumiflavin-3-acetic acid or 1,5-dihydrolumiflavin ($FlH_{2T} = FlH^- + FlH_2$) by formaldehyde are biphasic under all conditions of pH and concentration employed (μ = 1.0, 30.0 °C). The reaction is characterized by an initial "burst" of Fl_{ox} production which is followed by an apparent zero-order formation of Fl_{ox} until near the completion of the reaction. The kinetic time course is similar, but slower overall, than that observed for the reaction of ethyl pyruvate with FlH_{2T}.[3,9] The sequence of eq 3 has been established for the reaction of FlH_{2T} with pyruvic acid, pyruvate, pyruvamide, and ethyl pyruvate. What follows is a presentation of experimental results which add additional support to the general nature of eq 3 by establishing its validity for the reaction of formaldehyde with FlH_{2T}.

A typical absorbance vs. time plot for the oxidation of FlH_{2T}

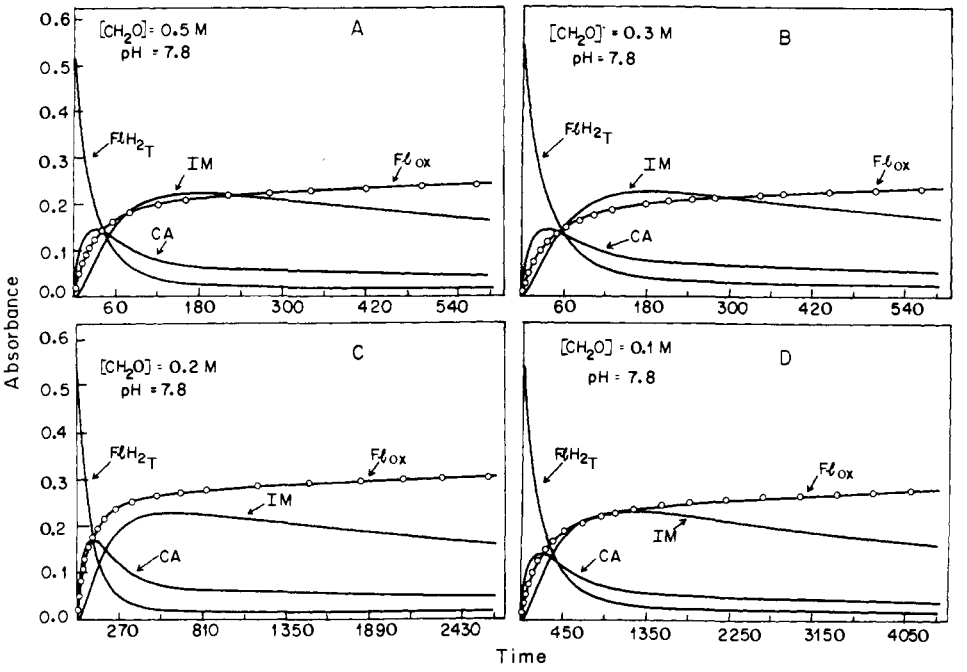
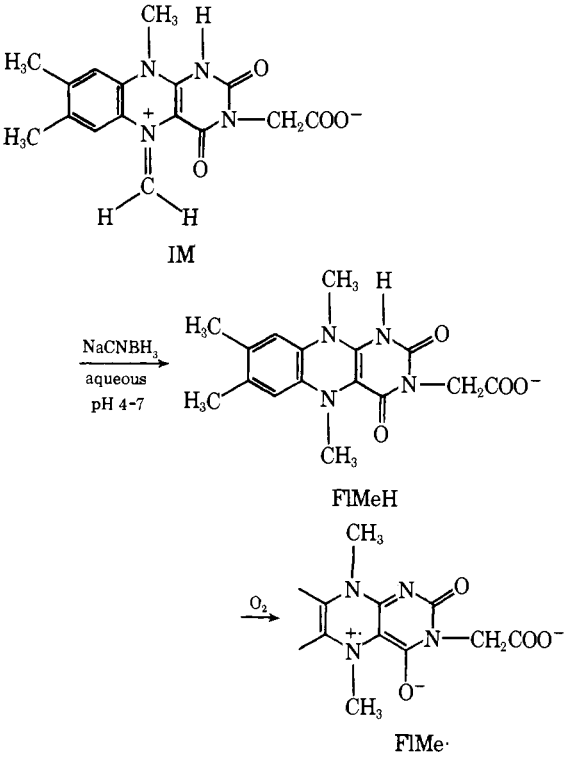


Figure 1. Absorbance (λ 443 nm) vs. time (min) plots for the reaction of formaldehyde (0.10–0.50 M) with 1,5-dihydrolumiflavin-3-acetic acid (5.0×10^{-5} M) at pH 7.8. The points are experimental and the solid lines are the theoretical analog simulation for the production and/or disappearance of starting materials, intermediates, and products according to eq 3.

by formaldehyde is shown in Figure 1. The points are experimental, and the lines are analog computer fitted[3] with the differential equations for the reactions of eq 3. The analog solutions for the reaction of formaldehyde with FlH_{2T} were as good (±0.005 OD units) as observed previously;[3] however, such fits, by themselves, do not establish the reactions of eq 3 conclusively. Figure 2 depicts the observed spectra after mixing formaldehyde (0.1 M) with FlH_{2T} (5×10^{-5} M). The spectra are very similar to that observed when ethyl pyruvate or pyruvic acid[3] serve as the carbonyl compound and is characteristic of an *N*(5)-alkyl-1,5-dihydroflavin (λ_{max} 345–360 nm).[18,19] As observed in the case of pyruvic acid et al.,[3] reduction of the imine (IM) to the *N*(5)-methyl-1,5-dihydrolumiflavin (FlMeH; λ_{max} 355 nm[11]) is readily accomplished in the pH range 4–7 with sodium cyanoborohydride (eq 7). The conversion of FlMeH to the blue flavin aminium cation radical (FlMe·; λ_{max} 585 nm) was achieved by addition of oxygen or ninhydrin[2,20] (3×10^{-4} M). The production of FlMeH on reduction with $NaCNBH_3$ establishes conclusively that both the 5-carbinolamine (CA) and imine (IM) are present. To date every carbonyl substrate investigated (except benzil[4]) has been shown, by this means, to produce CA and IM. Furthermore, in experiments (FlH_{2T} = 6.0×10^{-5} M, pH 6.0) performed with $NaCNBH_3$ (6×10^{-4} M) present in the reaction solution before addition of formaldehyde, there was observed an increase in absorbance at 443 nm of ~0.025 OD units. According to eq 3, this production of Fl_{ox} can only be due to reaction 3b. From the determined rate constants, k_1 and k_3, obtained from the analog computer simulation of eq 3, it can be calculated that an absorbance increase of 0.073 OD units should be obtained with only reaction 3b operative. Addition of $NaCNBH_3$ to the reaction mixture after completion of the initial burst always produced a concentration of reduced *N*(5)-methyl-1,5-dihydroflavin which, when added to the concentration of Fl_{ox}, produced during the burst accounted for 100% of the starting FlH_{2T}. These results are in agreement with previous observations[3] and conclusively show that reactions a and b of eq 3 account for the initial burst in Fl_{ox} formation. It may be concluded that the direct oxidation of FlH_{2T} by CH_2O (reaction b, eq 3) cannot involve the intermediacy of CA or IM.



(7)

With ethyl pyruvate and pyruvic acid as carbonyl substrates,[3] addition of formaldehyde after the initial burst resulted in a second small burst of Fl_{ox} formation. This established that a small concentration of free FlH_{2T} remained after the initial burst. With CH_2O as carbonyl compound addition of CH_2O following the initial burst did not result in an increase in the production of Fl_{ox} with time. This establishes that no more than a minute concentration of FlH_{2T} remains free. Figure 3 shows the addition of an aliquot of CH_2O 14.5 min

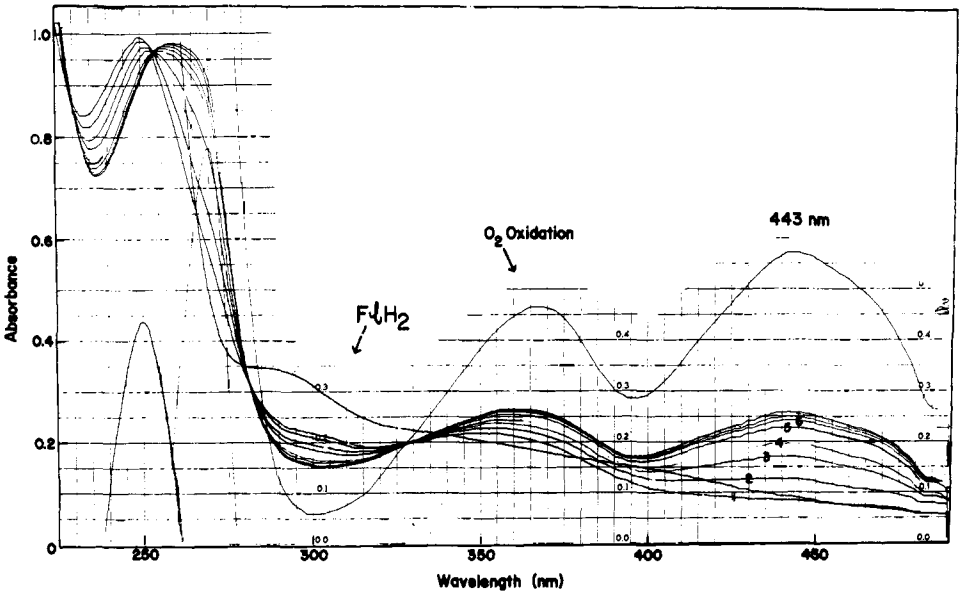


Figure 2. Spectral time study for the oxidation of 1,5-dihydrolumiflavin-3-acetic acid (5.0×10^{-5} M) by formaldehyde (0.10 M) at pH 6.77 (μ = 1.0): (0) 0 min (before mixing); (1) 0.5 min; (2) 1.5 min; (3) 4.0 min; (4) 6.5 min; (5) 16.5 min; (6) 25.5 min. Absorbance of CA may be seen at 350–355 nm.

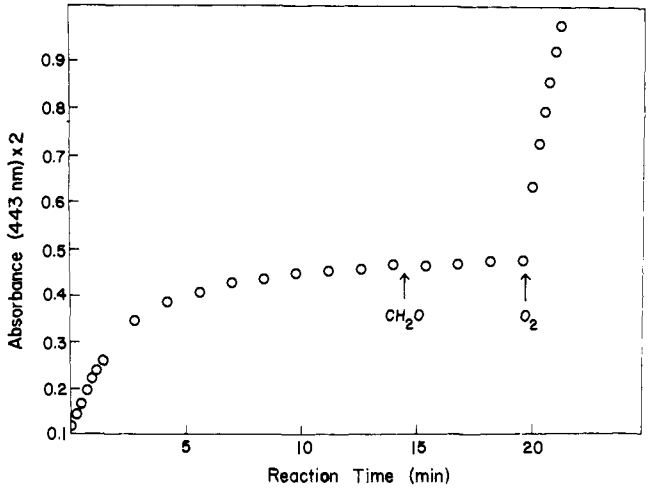
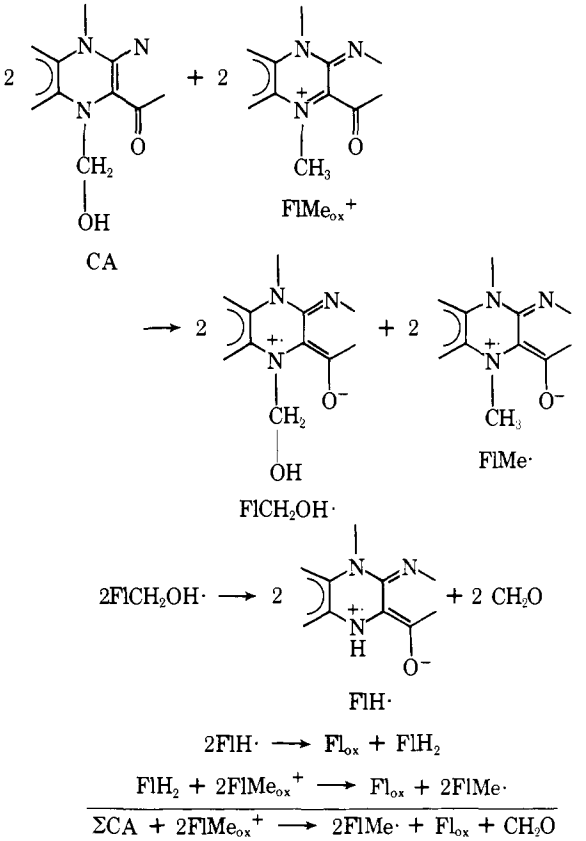


Figure 3. The effect of the addition of an additional aliquot of formaldehyde and of oxygen to a reaction mixture composed initially of 1,5-dihydrolumiflavin-3-acetic acid (6.0×10^{-5} M) and formaldehyde (0.1 M) at pH 6.25. Additions have been made following the initial burst. Formaldehyde was added after 14.5 min of reaction to give a final concentration of 0.2 M.

after initiation of a reaction of 0.1 M CH_2O with 6.0×10^{-5} M FlH_{2T} (pH 6.25, μ = 1.0). The final concentration of CH_2O was 0.2 M. Although only 30% of the initial FlH_{2T} has been converted to Fl_{ox} at the termination of the initial burst, no detectable concentration of 1,5-dihydroflavin remained as judged by a lack of increase in the production of Fl_{ox} (Figure 3). Analog simulation of eq 3 predicts that ~10% of FlH_{2T} will be present in the reaction mixture as free FlH_{2T}. This corresponds to an absorbance increase of 0.08 OD units which would not be detectable due to the formation of the $N(1)$-hydroxymethyl derivative (vide infra).

Additional support for eq 3 and the formation of $N(5)$-carbinolamine and $N(5)$-imine is also evidenced by the relatively slow return of Fl_{ox} on $^3O_2$ oxidation of the reaction mixture upon completion of the initial burst (Figure 3). The half-life for this reaction, $t_{1/2} \simeq$ 1.0–1.2 min, is essentially coincident with the observed rate of O_2 oxidation of 3,5-dimethyl-1,5-dihydrolumiflavin under similar conditions.[17]

Furthermore, the addition of 3,5-dimethyllumiflavin ($[FlMe_{ox}^+] = 8.2 \times 10^{-5}$ M) to a reaction mixture containing 0.15 M CH_2O and 6.4×10^{-5} M FlH_{2T} at pH 7.0 after the initial burst (126 min from initiation of the reaction) immediately produced the blue flavinium cation radical FlMe· (λ_{max} 585 nm) within mixing time (eq 8). Based on the extinction



(8)

coefficient of the flavin radical (ϵ 4200 at λ_{max} 585 nm),[20] the concentration of radical produced was 2.65×10^{-5} M. The difference between the total Fl_{ox} expected at the completion of the reaction and the amount of Fl_{ox} formed 126 min from initiation of the reaction is 3.80×10^{-5} M. Thus, all the re-

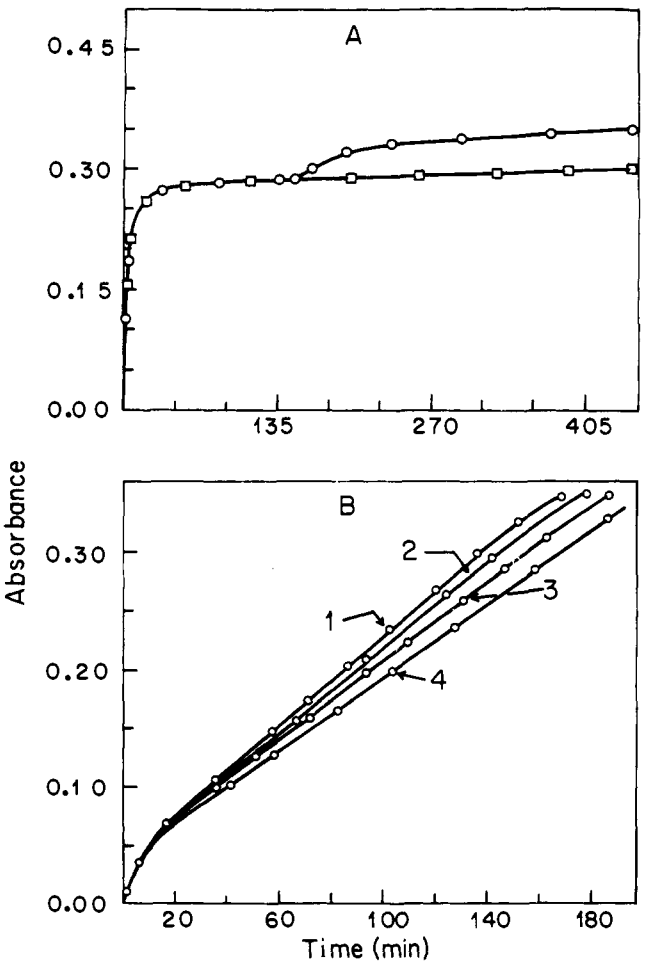
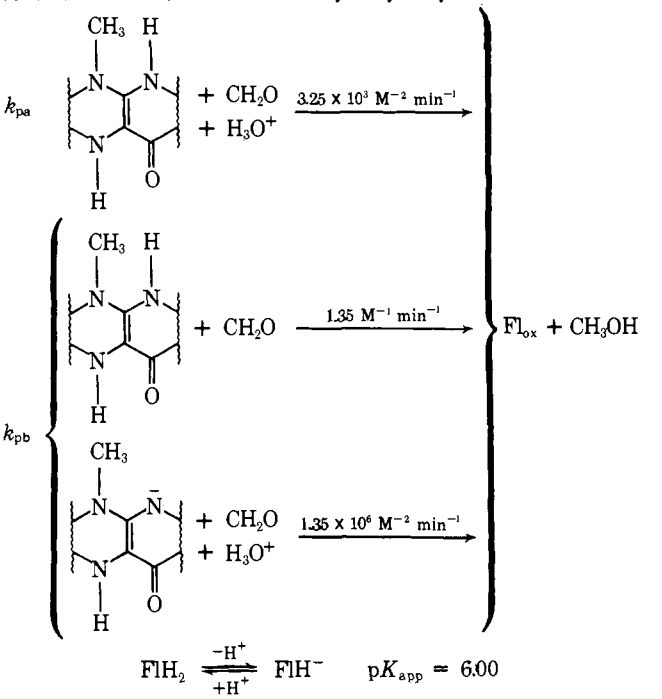


Figure 4. The effects of lumiflavin-3-acetic acid on the reaction of 1,5-dihydrolumiflavin-3-acetic acid (6.4×10^{-5} M) with formaldehyde (0.10 M) at pH 6.0 (λ 443 nm): (A) addition of an aliquot of Fl_{ox} (6.4×10^{-5} M) after 160 min of reaction; (B) addition of Fl_{ox} (2.1×10^{-5} M) at initiation of the reaction (20% methanol due to high Fl_{ox}): (1) 1.3×10^{-4} M Fl_{ox}; (2) 9.6×10^{-5} M; (3) 6.4×10^{-5} M; and (4) 3.2×10^{-5} M.

maining flavin ($2.65 \times 10^{-5} + 3.80 \times 10^{-5} = 6.45 \times 10^{-5}$ M) can be accounted for as being tied-up as CA and IM. That unoxidized FlH_{2T} is present in the form of N(5) adducts and not a 4a adduct is supported by the fact that radical production (FlR·) through comproportionation between N-alkyl-oxidized and -reduced forms has been shown to occur within mixing time.[20] On the other hand, comproportionation between 4a-hydroxy, 4a-methoxy, and 4a-alkyl derivatives of N(5)-substituted flavin and reduced N-alkyl flavin does not occur.[18,20,22] The oxygen reactivity of 4a adducts is also much less than in the case of N(5) adducts. Clerin and Bruice[21] have established that a 4a-indole adduct reacts with oxygen at a rate of ca. 10^{-4} min^{-1} and Ghisla et al. have shown that the 4a-methyl adduct of 3-methyllumiflavin is unreactive to oxygen under all conditions attempted (O_2, $O_2/h\nu$, $O_2/HNO_2/90°$, etc.).[18]

If the continuing production of Fl_{ox} following the initial burst is due to catalysis of the conversion of CA to FlH_{2T} plus aldehyde (reaction c of eq 3), then addition of Fl_{ox} would be expected to increase the rate of reaction following the initial burst while leaving the initial burst unaffected. Two types of experiments were performed to establish this catalysis by Fl_{ox}. Figure 4A shows the affect of the addition of an aliquot of Fl_{ox} (6.4×10^{-5} M) to the reaction of FlH_{2T} (6.4×10^{-5} M, pH 6.0) with 0.1 M CH_2O after completion of the initial burst (160 min from the initiation of the reaction). The apparent zero-order production of Fl_{ox} is accelerated. Figure 4B depicts the second experiment where the reaction of FlH_{2T} (6.4×10^{-5}

Scheme II. Reduction of Formaldehyde by Dihydroflavin



M, pH 6.0) with 0.1 M CH_2O is initiated with the presence of additional Fl_{ox} (3.2×10^{-5} to 1.3×10^{-4} M). The initial burst remains essentially unaffected while in the second phase the production of Fl_{ox} is accelerated. Clearly the reactions of Scheme II have been firmly established by the present and previously reported[3] observations.

Due to the necessary presence of methanol as a stabilizer in the formaldehyde solutions, the large concentration of formaldehyde over flavin present, and a lack of an adequate means to quantitatively determine methanol at $\sim 10^{-5}$ M, it was not possible to do a complete product analysis. However, based on electrochemical potential calculations (see ref 10), it is apparent that the reduction of Fl_{ox} by methanol will occur in basic solution at high methanol concentration due to the shift of the equilibrium, $FlH_{2T}/CH_2O \rightleftarrows Fl_{ox}/CH_3OH$ (K_e) to the left. At pH 10.9 and methanol concentrations varying between 1.5 and 9.2 M, the reduction of Fl_{ox} by methanol does occur. This is directly shown by the return of the Fl_{ox} absorbance (443 nm) upon oxygen addition to the reaction mixture at t_∞. The amount of FlH_{2T} produced may be assayed by this means and was found to be essentially identical (within 30%) with the amount of formaldehyde detected by differential pulse polarographic analysis of the reaction mixtures at $E_{1/2}$ = 1.67 V. Thus with methanol in excess, 1 mol of Fl_{ox} is converted to 1 mol of FlH_{2T} with the appearance of 1 mol of CH_2O. Table I presents the rates of reduction of Fl_{ox}, the concentrations of FlH_{2T} produced in the reaction as shown from the absorbance increase on oxidation with $^3O_2$, and the dependence of the concentration of formaldehyde, produced in the course of reduction, upon the initial concentration of methanol. It is evident that the overall reaction is indeed reversible under the proper conditions. Thus, by microscopic reversibility, methanol is the coproduct of the oxidation of FlH_{2T} by formaldehyde. The reduction of ethyl pyruvate and benzil by FlH_{2T} have previously been shown to yield the corresponding alcohol.[3,4]

The initial burst reaction is relatively large and well defined for the reaction of formaldehyde with FlH_{2T}, so it is possible to treat the apparent first-order rate constant for the burst (k_p) in the ordinary fashion for competitive formation of two products from a single reactant ($k_p = k_1 + k_3$). Following the initial burst, the change in absorbance (λ 443 nm) with time

Table I. The Production of 1,5-Dihydrolumiflavin-3-acetic Acid (FlH_{2T}) and Formaldehyde from the Reaction of Lumiflavin-3-acetic Acid (Fl_{ox}) plus Methanol[a]

[Methanol], M[b]	$[FlH_{2T}]$, M	$[CH_2O]$, M	k_r, min^{-1}
1.53[c]	2.40×10^{-7}	5×10^{-7}[f]	$\sim 6 \times 10^{-7}$
3.06[c]	3.20×10^{-7}	7×10^{-7}[f]	$\sim 7 \times 10^{-7}$
6.12[c]	1.01×10^{-5}	8.2×10^{-6}	2.35×10^{-5}
9.20[d]	1.53×10^{-4}	1.9×10^{-4}	3.41×10^{-5}
9.20[e]	1.72×10^{-4}	2.4×10^{-4}	3.18×10^{-5}

[a] $[FlH_{2T}]$ was obtained from the absorbance increase on addition of oxygen at the end of the reaction and $[CH_2O]$ was determined by differential pulse polarography. [b] pH 10.9 for the reaction mixtures. [c] $[Fl_{ox}] = 8.0 \times 10^{-5}$ M. [d] $[Fl_{ox}] = 8.3 \times 10^{-4}$ M. [e] $[Fl_{ox}] = 1.0 \times 10^{-3}$ M. [f] Unreliable due to the limits of polarographic detection of CH_2O.

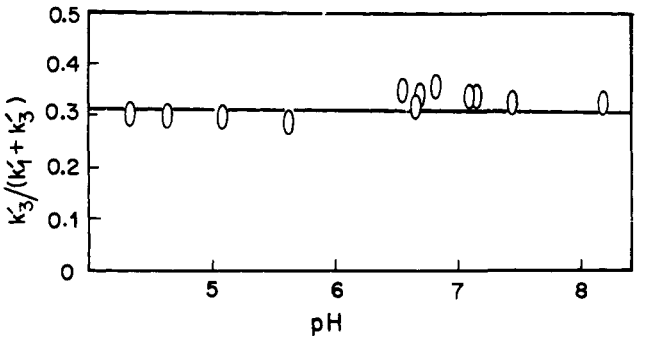
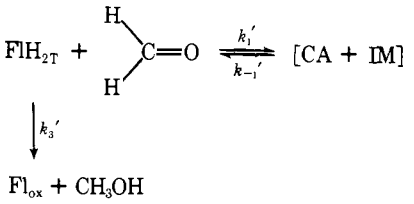


Figure 5. Plot of the ratio $k_3'/(k_1' + k_3')$ vs. pH for the reaction of 1,5-dihydrolumiflavin-3-acetic acid (7.0×10^{-5} M) with formaldehyde (0.10 M).

is virtually a straight line. Therefore, reactions a and b of eq 3 may be isolated and presented as in eq 9. Integration of this



(9)

kinetic scheme is easily accomplished by the application of Laplacian transforms. The intercept obtained by extrapolating the linear portion of the appearance of Fl_{ox} with time to $t = 0$ provides an absorbance value which when divided by the total Fl_{ox} absorbance equals $k_3'/(k_1' + k_3')$. Thus, the absorbance vs. time trace is approximated by the expressions

$$k_p = (k_1' + k_3')$$

$$k_z = \frac{k_3' k_1' k_{-1}'}{(k_1' + k_3')^2} [FlH_{2T}] \qquad (10)$$

where k_p represents the initial burst reaction and k_z corresponds to the linear apparent zero-order production of Fl_{ox}. The fraction of FlH_{2T} converted to Fl_{ox} at the termination of the initial burst reaction, $k_3'/(k_1' + k_3')$, was found to be essentially invariant over the pH range examined as shown in Figure 5 when [formaldehyde] = 0.10 M. The average value over 4 pH units is 0.32 ± 0.03. Thus the apparent first-order rate constants (k_3') for direct reduction of formaldehyde by FlH_{2T} is equal to 0.32 k_p (eq 11) at $[CH_2O] = 0.1$ M.

$$FlH_{2T} + CH_2O \xrightarrow{0.32\, k_p} Fl_{ox} \qquad (11)$$

An analog computer program wired for eq 3 would approximate a solution for the observed absorbance vs. time data for 30–40% of the overall reaction before breaking down due to the increasing importance of reaction c of eq 3. The complete reactions of eq 3 were necessary to obtain good fits of the kinetic points beyond 40% completion of reaction. As observed for the reaction of ethyl pyruvate with FlH_{2T} (which also has a linear apparent zero-order production of Fl_{ox} following the initial burst),[3] rate constants calculated from the simplified scheme of eq 9 agree to within ~15% of the rate constants obtained from the full analog simulation of eq 3. Figure 1 shows a composite of several typical analog fits (eq 3) of the reaction of FlH_{2T} with varying concentrations of formaldehyde at pH 7.8. A comparison of the values of k_1 and k_3 determined from the analog simulation to the calculated values of k_1' and k_3' determined by simply fitting the initial burst to the first-order rate law is made in Table II. Since the analog computer simulation provides results which are in relatively good agreement with the rate constants determined from k_p the latter procedure has been employed.

The log k_p vs. pH profile of Figure 6 has been fit by employing the kinetic expression of eq 12 which was derived from the reactions of Scheme II:

$$k_{obsd} = \frac{k_1 a_H^2 + k_2 K_a a_H}{K_a + a_H} \qquad (12)$$

From buffer dilution studies conducted at several pH values it can be concluded that the reaction of FlH_{2T} with CH_2O is insensitive to buffer catalysis. The very small positive slope of the buffer dilution plot for α-chloroacetic acid (plot A of Figure 7) at pH 1.77 cannot be interpreted to indicate general acid catalysis. Thus, if general acid catalysis were in effect, the weaker general acids would be most effective at $-\alpha = 0.9$, whereas if $-\alpha = 0.1$ all general acid species would exhibit catalysis.[23] One may conclude that the reduction of CH_2O by dihydroflavin species is a specific acid catalyzed reaction. Experiments with formaldehyde solutions, obtained com-

Table II. Comparison of the Rate Constants Calculated Using eq 9 (k_p, k_1', k_3', and k_{-1}') with k_1 and k_3 Obtained from the Complete Analog Simulation of eq 3 for the Reaction of FlH_{2T} (6.0×10^{-5} M) with Formaldehyde at pH 7.8

	Rate constants × 10^3, min^{-1}					
$[CH_2O]$M	k_p	k_1'	k_1	k_3'	k_3	k_{-1}'
0.01	1.35	0.815	0.698	0.535	0.502	0.150
0.02	2.73	1.52	1.51	1.21	1.14	0.209
0.03	4.01	2.24	2.58	1.39	1.56	0.295
0.05	5.05	3.33	2.93	1.72	1.67	0.450
0.10	5.67	3.98	3.45	1.70	1.70	0.182
0.20	15.7	9.98	7.75	5.72	4.68	0.184
0.33	29.9	20.6	21.2	9.11	9.03	0.761
0.50	32.4	21.1	25.3	11.3	12.0	0.633

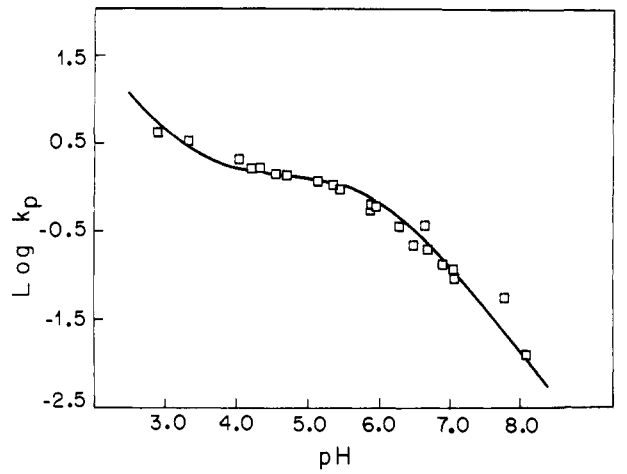


Figure 6. Plot of log k_p vs. pH for the oxidation of 1,5-dihydrolumiflavin-3-acetic acid (7.0×10^{-5} M) by formaldehyde (0.10 M). The theoretical line connecting the experimental points was generated by eq 12.

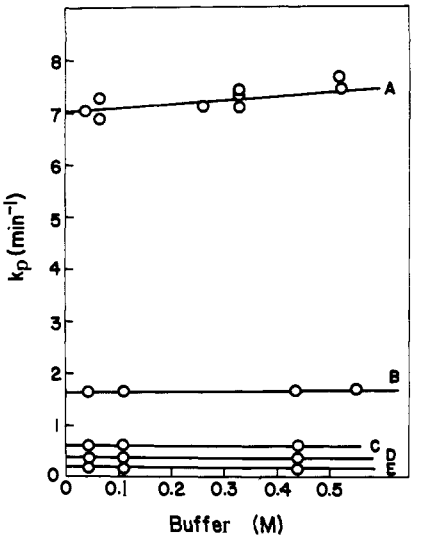


Figure 7. Effect of buffer concentration (μ = 1.0 with KCl) on the rate (k_p) of the burst reaction for the oxidation of 1,5-dihydrolumiflavin-3-acetic acid (6.0×10^{-5} M) by formaldehyde (0.10 M): (A) pH 1.77, α-chloroacetate; (B) pH 4.22, acetate; (C) pH 5.95, acetate; (D) pH 6.65, phosphate; (E) pH 7.31, phosphate.

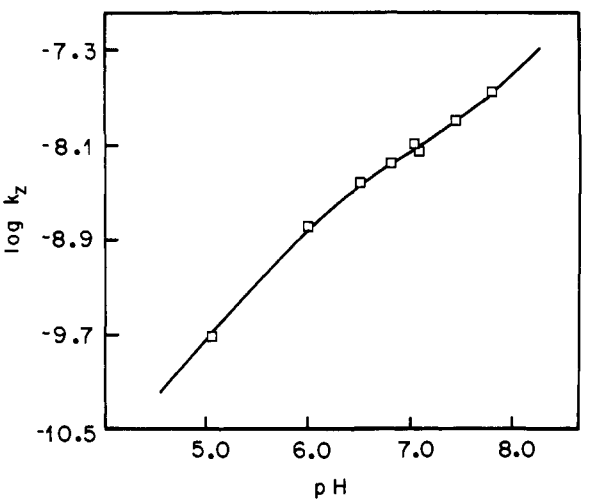


Figure 8. Plot of log k_z vs. pH for the second phase of the reaction of 1,5-dihydrolumiflavin-3-acetic acid (7.0×10^{-5} M) with formaldehyde (0.10 M). k_z is in units of M min^{-1} and the theoretical line connecting the experimental points was generated by eq 13.

mercially or prepared by pyrrolysis of paraformaldehyde, provided the same kinetic results.

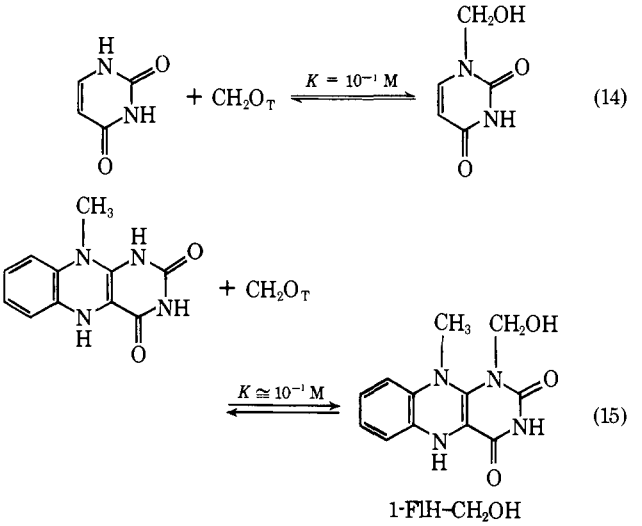
For the second and formaldehyde independent phase of the reaction the rate constants for formation of Fl_{ox} from carbinolamine-imine follow the relationship shown in the pH-log k_Z profile of Figure 8. The solid line connecting the data points of Figure 8 was generated from the expression of

$$k_Z = \frac{k_{Z1}K_w}{a_H}\left(\frac{a_H}{K_{app} + a_H}\right) + \frac{k_{Z2}K_wK_{app}}{(K_{app} + a_H)} \qquad (13)$$

where $pK_{app} = 6.61$; $k_{Z1} = 1.25 \times 10^{-1}$ M^{-1} min^{-1} and $k_{Z2} = 1.75 \times 10^{-2}$ M^{-1} min^{-1}. The values of k_Z were calculated from the zero-order slopes of the absorbance vs. time plots and converted to apparent first-order rate constants ($\epsilon = 1.2 \times 10^4$ for Fl_{ox}). Inspection of the kinetic equation for the formation of carbinolamine plus imine from FlH_{2T} in the initial burst (eq 12) and their conversion to Fl_{ox} in the second phase of the reaction (eq 13) reveals that: (a) the product (CA + IM) exists in acid and base forms as does the 1,5-dihydroflavin; (b) the formation of (CA + IM) from FlH_{2T} (as well as the direct production of Fl_{ox}) is acid catalyzed; and (c) the formation of Fl_{ox} from (CA + IM) in the second phase of the reaction is OH^- catalyzed.

The dependence of the rate constants, k_p and k_z, upon the concentration of formaldehyde was examined at two pH values (pH 5.75 and 7.80). As observed for the reaction of pyruvates[3] and as anticipated from eq 3, k_z was found to be independent of the concentration of CH_2O at either pH examined. At pH 7.80, a plot of k_p vs. $[CH_2O]$ was found to be linear with a zero intercept establishing that the initial burst reaction is first order in both $[FlH_{2T}]$ and $[CH_2O]_T$. After separating k_p into k_1 and k_3 the apparent second-order rate constants obtained from the slopes are 5.13×10^{-2} min^{-1} for k_1 and 2.49×10^{-2} M^{-1} min^{-1} for k_3 (Figure 9A,B). The kinetics of the reaction of formaldehyde with FlH_{2T} (k_p) at pH 5.75 differ from those observed with pyruvic acid, ethyl pyruvate, and pyruvamide in that k_p saturates markedly at high formaldehyde concentrations (Figure 9C). When k_1' and k_3' are separated from k_p both rate constants show similar Michaelis-Menten saturation kinetics (saturation occuring $\gtrsim$0.15 M CH_2O—not shown).

Saturation in $[CH_2O]$ at low but not high pH suggests that FlH_2 forms with CH_2O an inactive compound to an extent not seen with FlH^-. 1,5-Dihydroflavin may be looked upon as an elaborated uracil. Both possess NH pK_a values of ca. 7.0. Uracil has been established to form a hydroxymethyl derivative when undissociated.[24] Thus, the equilibrium reaction of eq 14 suggests eq 15.



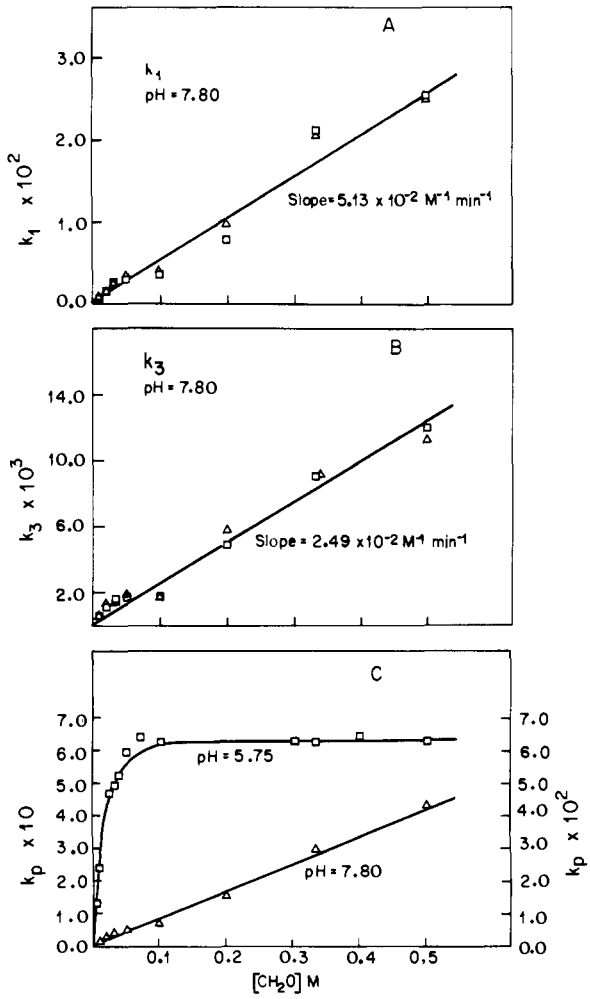
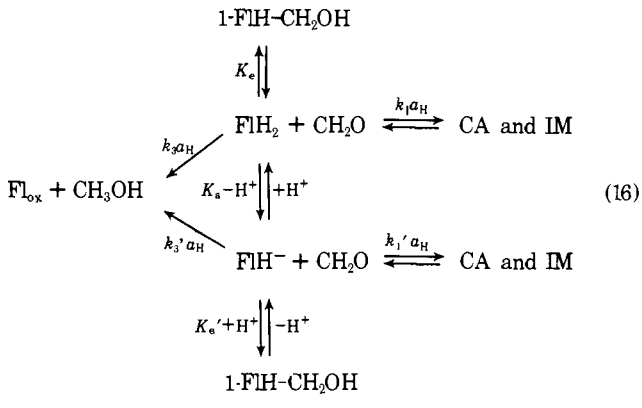


Figure 9. Dependence on initial burst reaction ($k_p = k_1 + k_3$) on the concentration of formaldehyde ([FlH_{2T}] = 6.0 × 10^{-5} M). In the plots of k_1 (A) and k_3 (B) vs. [CH_2O] at pH 7.8, the Δ are rate constants (min^{-1}) computed according to the simplified scheme (eq 9) and the □ are rate constants (min^{-1}) obtained from the analog computer solution of eq 3. The plot of k_p (min^{-1}) vs. [CH_2O] (C) shows a Michaelis-Menten like hyperbolic relationship at pH 5.75 (scale on left) and a linear relationship at pH 7.80 (scale on right).



(16)

The reactions of eq 16 may be considered for the initial burst. Assumption of material balance in F_T = FlH_2, FlH^-, and 1-FlH-CH_2OH provides

$$\frac{d[F_T]}{dt} = [CH_2O][F_T]\left\{\frac{(k_3 + k_1)a_H^2 + (k_3' + k_1')a_H K_A}{K_a + a_H(1 + K_e[CH_2O])}\right\} \quad (17)$$

At concentrations of CH_2O below saturation eq 17 reduces to

$$\frac{d[F_T]}{dt} = [CH_2O][F_T]\left\{\frac{(k_3 + k_1)a_H^2 + (k_3' + k_1')a_H K_a}{K_a + a_H}\right\} \quad (18)$$

which when combined with eq 10 and 11 yields

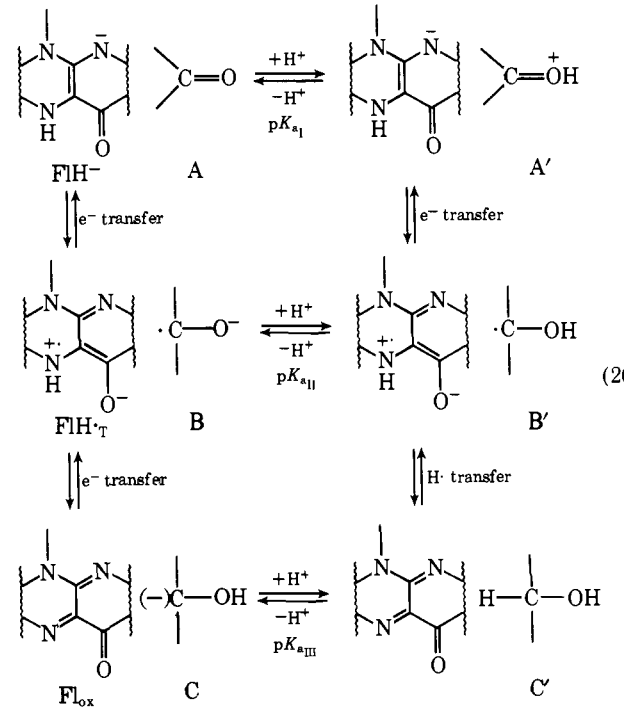
$$\frac{d[F_T]}{dt} = [F_T]0.32\left\{\frac{k_3 a_H^2 + k_3' a_H K_a}{K_a + a_H}\right\} \quad (19)$$

Reaction of FlH_2 with Several Carbonyl Compounds. The reduction of a selected number of other aldehydes and ketones by FlH_{2T} was investigated at two pH values under anaerobic conditions. The results listed in Table III establish that a wide variety of ketones and aldehydes are reduced by 1,5-dihydroflavin.

Discussion

A common kinetic sequence (eq 3) has been established for the 1,5-dihydroflavin reduction of a series of carbonyl compounds which includes pyruvic acid, pyruvate ion, ethyl pyruvate, pyruvamide,[3] benzil,[4] and from this study formaldehyde. The pH dependence of the $2e^-$ transfer step leading from >C=O + FlH_{2T} → H—C—OH + Fl_{ox} has been determined for pyruvic acid, pyruvate, and formaldehyde. The results establish the reaction to be acid catalyzed. For the reduction of pyruvic acid both H_3O^+ and pyruvic acid (pK_a = 2.2) serve as catalysts[3] while the reduction of formaldehyde is specific acid catalyzed. The reduction of benzil, on the other hand, is pH independent between pH ~2 and ~8.[4]

Arguments have been presented (see Introduction and ref 3) which establish that radical mechanisms are allowed for reduction of carbonyl compounds by 1,5-dihydroflavin. The following alternate routes (eq 20) may be considered[25] for



(20)

reduction of carbonyl compounds by FlH^- (a completely analogous mechanism may be written for FlH_2). The mechanism of radical intermediate formation will be dependent upon the standard free energies of formation of >C=$^+$OH as compared with ⁓FlH··C-O^-⁓ and ⁓FlH··C—OH⁓ and the means of product formation will be dependent upon $pK_{a_{III}}$. For the interconversion: benzoin + Fl_{ox} ⇄ benzil + FlH_{2T}, the reaction sequence is proposed to be simply A → B → C (eq 20). This may be appreciated by the facts that (1) pK_{a_I} for benzil may be approximated as −7; (2) the reduction of benzil by

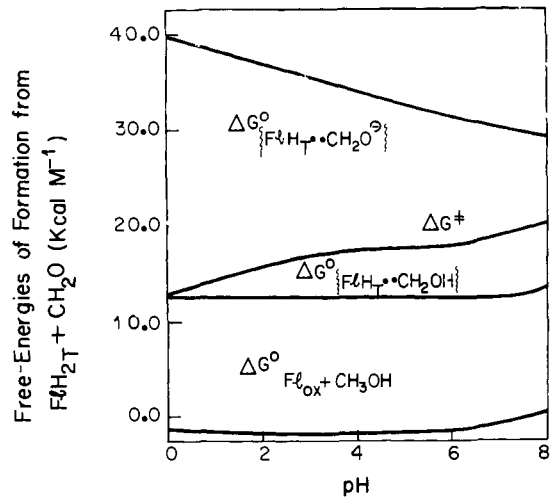


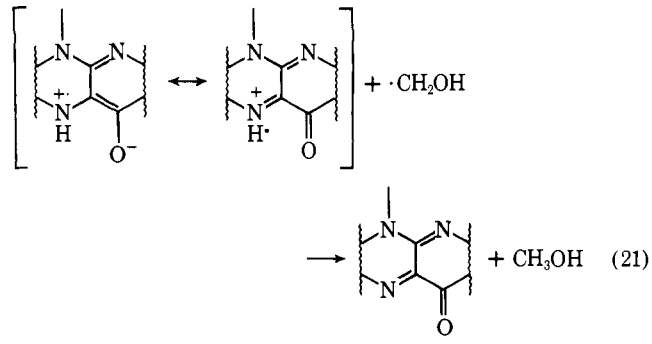
Figure 10. Employing the initial state of FlH_{2T} (1 M) and CH_2O (1 M) the free energies ($\Delta G°$) of formation of plausible radical intermediates and products (at 1 M) are plotted vs. pH. The pH dependence of the $\Delta G°$ values may be compared to the pH dependence and level of $\Delta G^{\ddagger}$ for the reduction of formaldehyde by 1,5-dihydrolumiflavin-3-acetic acid.[28]

Table III. Reaction of FlH_{2T} with Several Carbonyl Compounds[a]

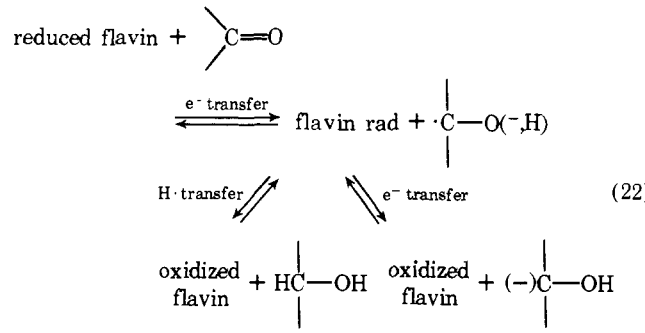
Aldehyde and ketone	k_r, M^{-1} min^{-1} pH 5.14 ± 0.06	pH 8.85 ± 0.05
Formaldehyde[b]	11.8	0.0123[c]
Chloral	0.0181	0.310
Pyridine-4-carboxaldehyde	30.9	0.379
Glyoxal	0.272	0.640
Phenylglyoxal[d]	137	151
Pyridoxal[e]	3.1	24.2
Glycoaldehyde	0.923	*g*
Glyoxylic acid, hydrate	0.212	
Oxalacetic acid	0.142	*h*
Glycolamide		*h*
Dichloroacetaldehyde	0.0082	
Monochloroacetaldehyde	0.0021	
Acetaldehyde	*h*	*h*

[a] 30 °C, μ = 1.0 M KCl, 10 vol % methanol. $[FlH_{2T}] = 5.0 \times 10^{-5}$ M, [aldehyde or ketone] = 0.10 M. [b] The rate constants listed were from the rapid initial reactions. [c] pH 8.10. [d] [Phenylglyoxal] = 0.010 M. [e] [Pyridoxal] = 5×10^{-3} M. [f] See ref 3. [g] Reacts faster with Fl_{ox} to produce FlH_{2T} at basic pH. [h] No reaction.

1,5-dihydroflavin is not acid catalyzed; (3) pK_{all} has been determined to be 5.5;[26] and (4) it has been shown that the benzoin carbanion is the immediate substrate in the reduction of Fl_{ox} by benzoin.[4] The reduction of formaldehyde to methanol by 1,5-dihydroflavin represents the other extreme. Since the reaction has been shown to be specific acid catalyzed, the sequence is A → A′ → B′ → C′ (eq 20). This conclusion is dictated by the high free-energy content of the species $\cdot CH_2O^-$ and $(-)CH_2OH$ which precludes states B and C from forming along the reaction path (Figure 10). Thus, at pH 7.0 the standard free energy of formation of B from A ($\Delta G°_{A \rightarrow B}$) exceeds the standard free energy of formation of B′ from A ($\Delta G°_{A \rightarrow B'}$) by ~13 kcal M^{-1} and $\Delta G^{\ddagger}_{expt}$ by 12–13 kcal M^{-1} (for calculations of $\Delta G°$ values see ref 10). For these reasons states B and C cannot occur along the reaction coordinate in the reduction of H_2CO by FlH_2 or FlH^-. Conversion of B′ to C′ is proposed to involve H· transfer from FlH· to $\cdot CH_2OH$ (eq 21). For the reduction of pyruvic acid and derivatives (CH_3CO-COX where X = $-O^-$, -OH, $-NH_2$, and -OEt) the standard free energies of formation of A′, B, and B′ are less than $\Delta G^{\ddagger}$ so that all three states are allowed along the reaction coordinate.[3] Further, from the pH dependence of $\Delta G^{\ddagger}$, it has been concluded that state C is also allowed providing the microscopic pK_a of the α-H of $CH_3CH(OH)COX$ (X = -OH, $-O^-$, -OEt, $-NH_2$) does not exceed ~25.[3]



In conclusion, two radical paths appear reasonable for the overall oxidation reduction of flavins by carbonyl compounds. Ignoring protonic equilibria these are given in eq 22:



The importance of H· and $1e^-$ transfer to yield oxidized flavin from the radical pairs is dependent upon the pH and the pK_a of the carbon acid (alcohol). Though N(5) adducts (carbinolamines) are not intermediates for the reduction of formaldehyde, pyruvic acid, pyruvate, ethyl pyruvate, and pyruvamide by 1,5-dihydroflavin, this possibility cannot be eliminated for the flavin mediated interconversion of benzoin to benzil (see discussion in ref 4).

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Carbon-13 NMR Studies on Cholesterol Biosynthesized from [^{13}C]Mevalonates

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Abstract: Two specimens of [^{13}C]cholesterol, one biosynthesized from [5-^{13}C]mevalonate and the other from [3′,4-$^{13}C_2$]mevalonate, were examined by Fourier transform ^{13}C NMR spectroscopy. The observations confirmed the biochemically predictable positions of ^{13}C labels in cholesterol and provided also an unambiguous proof of the assignments of the ^{13}C resonances for 16 of the 27 carbon atoms in the ^{13}C NMR spectrum of cholesterol. New and additional evidence is provided for a 1:2 methyl shift, from position 14 to 13, during the cyclization of squalene oxide to lanosterol. The stereochemistry of the reduction of the 24(25) double bond in the side chain of lanosterol (or of desmosterol), determined by others, and the magnetic nonequivalence of C-26 and C-27 in cholesterol led to the assignment of the resonance at 22.9 ppm downfield from the reference to C-27 (derived from C-3′ of mevalonate), the pro-(*S*)-methyl group attached to C-25. The resonance of C-26, the pro-(*R*)-methyl group attached to C-25, originating from C-2 of mevalonate, is at 22.7 ppm downfield from the reference. One-bond (sp^3) and triple-bond (vicinal) ^{13}C–^{13}C coupling constants are also reported.

During the earliest explorations of cholesterol biosynthesis in the 1950's the time-consuming carbon-by-carbon degradations of cholesterol[2] or squalene biosynthesized from [^{14}C]acetates[3a] or [^{14}C]mevalonate[3b,c] were the only means available to establish overall biosynthetic pathways. The availability of ^{13}C NMR would reduce today similar studies to a few weeks instead of the years of effort needed earlier.

The intermediary steps in the biosynthesis of cholesterol starting from acetyl-CoA are well known and the positions of the two carbon atoms of acetate in the sterol have been completely mapped.[2] When, after the discovery of mevalonic acid as an intermediate in sterol biosynthesis by Tavormina et al.,[4] Cornforth et al.[3b] determined the distribution of mevalonate carbons in squalene, they also predicted the probable distribution of the carbon atoms of this intermediate in lanosterol and cholesterol. Isler et al.[5] have shown that C-2 of mevalonate was found at C-7, C-22, and at either C-26 or C-27 of cholesterol, in accord with the prediction.

Although the distribution of the carbon atoms of mevalonate in cholesterol has been determined for only a few positions, the total pattern cannot be in doubt because (a) of the established pattern of acetate carbons in cholesterol; (b) of the known biosynthesis of mevalonate from acetyl-CoA through 3-hydroxy-3-methylglutaryl-CoA; (c) of the well-documented biosynthesis of squalene from mevalonate; and (d) because the principal postulates of the "biogenetic isoprene rule", pertaining to the cyclization of squalene (2,3-dioxide) to lanosterol, formulated on theoretical grounds by Eschenmoser et al.,[6b] following the proposals of Woodward and Bloch,[6a] have been proved also. The "biogenetic isoprene rule" requires—among other things—two 1:2 methyl migrations during the cyclization of squalene to lanosterol: one from C-14 to C-13 and the other from C-8 to C-14. The first of these was proved by Cornforth et al.[7] with the aid of [3′,4-$^{13}C_2$]mevalonate and the second by Maudgal et al.[8] with the aid of a synthetic all-trans [^{13}C]squalene. The first of these methyl migrations is of particular interest because it occurs within one specific isoprenoid unit of squalene and results in two ^{13}C atoms being bonded together (C-18/C-13) in cholesterol when [3′,4-$^{13}C_2$]mevalonate, diluted with unlabeled mevalonate, is the starting substrate.[7]

We have examined by ^{13}C NMR two specimens of cholesterol, one biosynthesized from [5-^{13}C]- and the other from [3′,4-$^{13}C_2$]mevalonate. The spectra obtained confirmed the expected distribution of excess ^{13}C in cholesterol and provided an independent check on the assignments of the ^{13}C resonances in cholesterol as well as values of certain ^{13}C–^{13}C coupling constants.

The assignments of the resonances in the ^{13}C NMR spectrum of cholesterol were originally made by Reich et al.[9] and confirmed by Mantsch and Smith,[10] but the resonances of C-12, C-16, and C-20 were only tentatively assigned. Subsequent work[11,12] showed that the original assignments of the resonances of C-12 and C-16 should be reversed.[13] The ^{13}C NMR spectra of biosynthetic [^{13}C]cholesterols provided an independent confirmation of the revised assignments.

Experimental Section

[^{13}C]Cholesterols. Two specimens of [^{13}C]cholesterol were obtained by biosynthesis in vivo in 14-day-old rats (about 20-g body weight) injected over 2 days subcutaneously with four nearly equally spaced doses of [^{13}C]mevalonates (see below), 1 μmol/g of body weight at each injection. The animals were killed 16 h after the last dose and the cholesterol was prepared from the carcasses and organs (excepting